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13. ABSTRACT *(Maximum 200 words)*

This project aimed to study a novel gene, ADAMx, which was found in human breast tumor MCF-7. Partial sequence of ADAMx reveals that it constitutes domains characteristic of the ADAM proteins. Furthermore, ADAMx appears to be the human analog of fertilin-a. So far, experiments have focused on DNA cloning and antibody production.

Unfortunately, upon further study, ADAMx was found to be a pseudogene as marked by stop codons at the 5'-end of the transcript and an insertion and deletion sequences in the region. This result was confirmed and published by another group of scientists. This is a rather unexpected result for this proposal and for my research career. However, for the scientific community as a whole, it would still be interesting to conduct research in order to interpret this result, especially from evolution point of view.

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Table of Contents

Please be advised that this final progress report summarizes preliminary data from the study "An ADAM Protein In Breast Cancer". A revised SOW has was also submitted.

Summary

The hypothesis of this proposal is that a novel ADAM protein, ADAMx, may play a role in the interaction between cell surface integrins and ECM protein. Particularly, the disintegrin domain of ADAMx may alter adhesion and migration properties of cells and consequently, ADAMx may be important in tumor metastasis. The goal of this proposal is to clone and express the full length ADAMx and characterize its function in breast carcinoma cells.

This report summarizes the identification, cloning of ADAMx gene and production of anti-disintegrin domain antibody. Approximately 80% of the gene was cloned and sequence alignment with known ADAMs suggested that ADAMx is a human analog of fertilin-α, an ADAM protein found in many other animals such as mouse, monkey and guinea pig and is involved in sperm-egg fusion. However, we found that this fertilin-α human analog is a non-functional pseudogene mainly because of the existence of stop codons in the 5'-region of the transcript. This conclusion is consistent with a study reported by Hall and coworkers (24).

I. Introduction

The cell-extracellular matrix (ECM) interactions are coordinated series of events composed of modification of cell-cell binding and cell-ECM adhesion, proteolytic degradation of ECM and cell de-attachment, migration through ECM and entering into the circulation. When unchecked, these interactions result in over-proliferation, circumvention of apoptosis and thus tumor growth and metastasis Surface integrins, Matrix metalloproteases (MMPs) and membrane-type metalloproteases (MT-MMPs) constitute one of the pivotal points of ECM turnover (1-8). MMPs and MT-MMPs are intimately involved in the malignant phenotype as shown by numerous studies including those using the human breast carcinomas. This current study focuses on a newly cloned membrane protein, ADAMx, which contains both a metalloprotease domain and an integrin-binding domain.

The ADAMs are a family of complex membrane proteins which mostly resembles the MT-MMPs (8). ADAMs are usually composed of a pro-protein domain (PP), a metalloprotease domain (MP), a disintegrin-like domain (DIS), a cysteine-rich domain (CR), a transmembrane domain (TM) and a cytoplasmic tail (CT). Some of the ADAMs also have EGF-like motifs (EGF) and fusion peptides (FP). To date, about 20 members of the ADAM family proteins are cloned from a variety of species and tissues (9-11).

ADAMs have been implicated in fertilization in the aspect of cell-cell fusion, especially, the sperm-egg fusion (9,11-13). ADAMs are also implicated in myoblast differentiation and myotube formation (14). Another interesting function of the ADAMs is proteolysis of degradation and remodeling of surface proteins and ECM components, as the closely related snake venom metalloproteases (SVMP) do (15). One study showed that TACE (tumor necrosis factor-α converting enzyme), an ADAM protein, can induce the release of TNF-α from tumor cells, presumably by cleaving membrane-bound TNF-α precursor (16). Another ADAM, ADAM-11, is a candidate human cancer tumor suppresser, as its gene is disrupted in both the breast and ovarian cancers by somatic re-arrangements (17). In conclusion, the ADAM family proteins are likely involved in interactions with ECM components and with cell surface proteins, including the integrins. Therefore they most likely play functional role in tumorigenesis.

I have cloned a novel ADAM protein (ADAMx) from non-invasive human breast epithelial cell line MCF-7. ADAMx is not found in the MDA-MB-231 or MDA-MB-435 human breast

carcinomas which have mesenchymal (fibroblast-like) morphology and are highly invasive (18,19). This expression pattern is opposite to that of the MMPs and MT-MMPs, both of which are found to be expressed in the invasive cells, but not in the MCF-7 cell (20). The difference in the expression patterns leads to the hypothesis that ADAMx, as part of the ECM remodeling machinery, may act through a pathway different from that of the MMPs and MT-MMPs. It also suggests that ADAMx may be important for maintaining breast cell epithelial morphology and that the loss of ADAMx could link to tumor progression and metastasis.

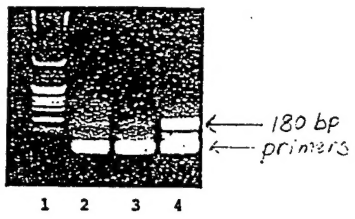
II. Results

II.1. Identification of ADAMx from Breast Cancer Cell lines

Homology PCR was used to identify ADAMs in several human breast cancer cell lines, including MDA-MB-435 , MDA-MB-231 and MCF-7. The 435 cells represent advanced tumors because they are highly invasive, the 231 cells are moderately invasive and the MCF-7 cells are not invasive; Thus the latter cell line represents early stage tumors (18,19).

The initial cloning was performed by homology RT-PCR method using total RNA from the three cell lines, oligo dT_{20} and a pair of degenerate primers derived from conserved sequences within ADAMs' disintegrin domains. Figure 1 shows a result of a RT-PCR.

Figure 1, lane 1, 1Kb DNA ladder; lane 2, 435 cell RNA; lane 3, 231 cell RNA and lane 4, MCF-7 cell RNA. Each sample lane contains one fifth of the PCR reaction volume. The expected size of the PCR product is ~ 180bp which is marked with the arrow. The lower band in the gel was resulted from the primers added in the reaction. This is a representative of three RT-PCR experiment repeats.



Sequence analysis revealed that the MCF-7 cell RNA contains one novel ADAM protein. I designated this novel ADAM as ADAMx. In addition, human homologue meltrin-α and metargidin was also found in MCF-7 cells. Several ADAMx gene-specific primers were synthesized according to the sequence information derived from these experiments. RT-PCR using the gene-specific primers for ADAMx confirmed that there was no detectable ADAMx messenger in the invasive 231 and 435 cells (not shown). ADAMx is also identified in a human osteosarcoma cell line (MG63, not shown).

II.2. Cloning Full Length ADAMx Gene

I used two approaches to clone the full length ADAMx gene. One was the Rapid Amplification of cDNA ends (RACE) (21,22) and the second is to screen the human cDNA libraries.

Two human DNA libraries were screened. One is an human placental cDNA library (Invitrogen) and was screened by the filter-lifting method. The other one is a commercial human BCA library (Research Genetics, Huntsville, AL) and it was screened by using the high density membrane hybridization method. The [α]-^{32}P-radio-labeled DNA probes used for both libraries were synthesized using the Random Primed DNA Labeling Kit (Boehringer Mannheim) and the dsDNAs of ADAMx encode either the disintegrin domain of ADAMx or the disintegrin domain plus 5' end of the cysteine-rich domains. Unfortunately, none of the libraries gave positive clones. This is an indication that ADAMx might be a rare gene.

RACE (Rapid Amplification of cDNA Ends) was used in order to clone the rest of the sequence of ADAMx gene. As a result, at lest 80% of the ADAMx gene was cloned (this is

based on the sequence comparison to other known ADAM genes). The sequence showed that ADAMx contains a pro-domain, a metalloprotease domain represented by the presence of the conserved Zn^{2+}-binding peptide sequence HELGHNLGIQH; The MP domain is followed by a disintegrin domain, a cysteine-rich domain, an EGF-like domain and a transmembrane domain (Figure 2). The TM region is predicted by hydropathy plot analysis (Figure 3).

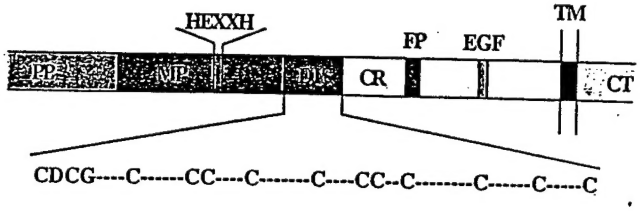


Figure 2. Domain arrangement of ADAMx

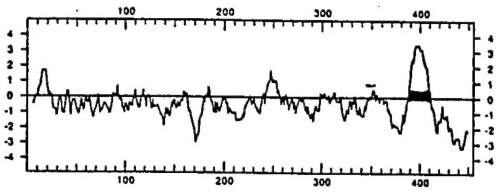


Figure 3. Kyte Doolittle hydropathy plot of ADAMx (partial sequence). The potential fusion peptide is marked by an arrow and the predicted transmembrane region is blackened.

An alignment of the putative integrin-binding loop of several ADAM's disintegrin domains is shown in figure 5. Note that in ADAMx, RGD sequence in metargidin (ADAM-15) is replaced by a SRS sequence.

Figure 4. Sequence alignment of "integrin binding loop" among several ADAMs. Here MG63/MCF7 represents ADAMx gene.

Metargidin	-CRPT**RGD**C-
MG63/MCF-7	-CCPSSRSC-
Human Meltrin-α	-CRDSSNSC-
Macaque Fertilin-α	-CRPTQDEC-

The confirmed protein sequence of ADAMx is shown in figure 5 (below).

....LCQHPALWKNQVALEEAKIKFQTWAPQKWNLRLGLVPGPSCIRLEILMLLVIFVPSMYC
HLGSIYYSFYEIIIPKRLTVQGGDSPVEGLSYLLLMQGQKHLVHLKVKRNHFVNNFPVYSY
HNGLLGQESPFISHDCHYEGYIEGMSGSFVSVNICAGLRGTSSLRRKNLTALSPWTLQD
GLNMCYTPWHIKRESPVVSTSWQQGSRKPHDLQALSYLCSHKKYVEMFVVVNNQRFQ
MWGSNVNETVQTVVDIALANSFTRGINTEVVLAGMEIWTEGDLIDVTVDLQITLRNFNHW
RQEMFFHRAKHDVAHMIVGHHPGQNMGQAFLSGACSSGFAAAVESFHHEDVLLFAALM
AHELGHNLGIQHDHSACFCKDKHFCLMHENITKESGFSSCSSDYFYQFLREHKGACLFN
KPRPRSRKRRD**SACGNGVVEDTEQCDCGSLCQHHACCDENCILKAKAECSDGPCCHK**
CKFHRKGYPCCPSSRSCDLPEFCNGTSALCPNNRHKQDGSKCHTIYECLKVHCMDPNN
QCLQLYGYGAKSASQECYNSMNSKGDQFGNCGISTSPGSQYVRCSDGNIFCGKLICSGI
TGLPKINLQHTMIQVPQGDGSCWSMDAYMSTDIPDEGDVHNGTYCAPNKVCLNSACTD
KTPVISACNPKKTCNGKGVCNDLGHCHCNEGHAPPDCVTAGSGGSVDSGLPGKLGGTP
SGEGENHNMTHSRREEHAVDMMI*LSFIILFIILLLSTII**SACLKNHQRLPRQKFLQQWLHH
RPQK*SQKQQKWPQKKKKKHMPWT

Figure 5. The sequence of ADAMx (incomplete). The zinc-binding motif in the metalloprotease domain is double underlined. The disintegrin domain is in bold. The potential fusion peptide is underlined and the putative transmembrane region is in italics. The 3' coding region, 5' coding region and untranslated region of the gene remain to be completed.

The functional motifs/domains of ADAMx were identified by comparison of the sequence to those of known ADAMs. Sequence homology search using the NCBI BLAST Search showed

that ADAMx has the highest protein sequence homology with the fertilin-a molecules (ADAM-1) from macaque, mouse and guinea pig (23). In some regions, ADAMx's amino acid sequence is at least 70% identical to the fertilin-a of macaque. An alignment of partial sequence of ADAMx with fertilin-α molecules is shown (see attachment). Therefore, ADAMx seems to be a human homologue of fertilin–α.

II.3. Polyclonal antisera production

I have raised polyclonal antibodies against the disintegrin domain of ADAMx. To make the antigen, the DNA of DIS domain was PCR amplified with primers each containing a restriction enzyme cleavage site. The PCR product was cloned into expression vector pET15b (Novagen) using restriction sites Xho I and BamH I. The protein was expressed in *E. coli* bacterial BL21 (DE3) cells upon IPTG induction. The recombinant protein was found to be retained in the inclusion bodies and thus had to be solubilized by using 6M urea. The protein contains a N-terminal His-tag and was coupled to a Ni^{2+}-affinity Sepharose 6B column (Figure 6). The protein was eluted off using 1M imidozole in 6 M urea/PBS. The sample was dialyzed against 6 M urea/PBS to remove imidozole. The purified and concentrated antigen was used to immunize rabbits. The ability of the antisera to recognize recombinant disintegrin domain of ADAMx was confirmed by ELISA. The disintegrin domain of ADAM-15 was used to examine cross reactivity (Figure 7).

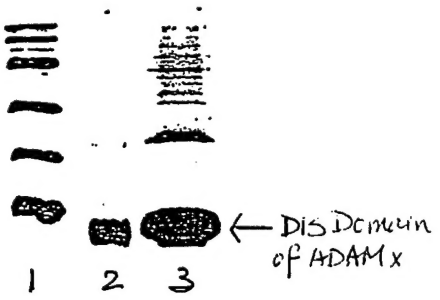


Figure 6. 15% reducing SDS-PAGE analysis of His-tagged ADAMx disintegrin protein purified by Ni^{2+} resin chromatography. Lane 1, protein markers; Lane 2 and 3, ADAMx eluted from the affinity column.

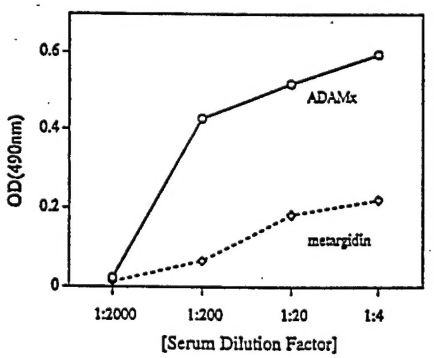


Figure 7. ELISA measurement of the specificity and activity of polyclonal antibodies raised against the disintegrin domain of ADAMx. The disintegrin domain of ADAM-15 metargidin, was used as a control Recognition of the antigens was accessed by optical density at 490nm for the reaction of *o*-phyenylenediamine.

III. ADAMx appears to be an expressed pseudogene

Upon further cloning the 5'-end sequence of ADAMx, several ambiguous sequences showed up in the 5' region that resulted in several possible DNA reading frames. None of the reading frames, unfortunately, gave open reading sequence for the gene. In addition, there is a two amino acid insertion and a 75 amino acid deletion in ADAMx, the human fertilin-α analog, as compared to the sequences of fertilin-α from macaque, mouse and guinea pig (see attachment). These results were quite bothersome.

In the process of performing more experiments to verify the above sequence information, a similar work was published by Hall's group, stating that the human fertilin-α is a non-functional pseudogene and that this is the only copy of human fertilin-α (24). Their human fertilin-α turned out to be the same gene as ADAMx. This study confirmed my suspicions that ADAMx is a pseudogene with expressed transcript.

IV. Conclusion

This project aimed to study a novel gene, ADAMx, which was found in human breast tumor MCF-7. Partial sequence of ADAMx reveals that it constitutes domains characteristic of the ADAM proteins. Furthermore, ADAMx appears to be the human analog of fertilin-α. So far, experiments have focused on DNA cloning and antibody production.

Unfortunately, upon further study, ADAMx was found to be a pseudogene as marked by stop codons at the 5'-end of the transcript and an insertion and deletion sequences in the region. This result was confirmed and published by another group of scientists. This is a rather unexpected result for this proposal and for my research career. However, for the scientific community as a whole, it would still be interesting to conduct research in order to interpret this result, especially from evolution point of view.

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